Evaluation of Ascorbic Acid as a Quorum-sensing Analogue to Control Growth, Sporulation, and Enterotoxin Production in *Clostridium perfringens*

J.S. NOVAK AND P.M. FRATAMICO

ABSTRACT: Inhibition of quorum sensing measured by autoinducer-2 (AI-2) activity was investigated in the presence and absence of ascorbic acid, an AI-2 analogue. Subsequent effects on AI-2 production, as well as growth, sporulation, and enterotoxin (*C. perfringens* enterotoxin [CPE]) production in *Clostridium perfringens* were examined. The addition of ascorbic acid to supernatants from ground beef resulted in a 100-fold decrease in AI-2 activity. The addition of sodium ascorbate, a nonacidic salt of ascorbic acid, also resulted in AI-2 assay inhibition. Spore production decreased in the presence of ascorbic acid. Western immunoblot analyses showed that CPE levels were highest after 24 h without ascorbic acid. This study explored the unique concept of signal inhibition to control pathogens in food.

Keywords: ascorbic acid, inhibition, *Clostridium perfringens*

Introduction

The presence of the ubiquitous spore former, *Clostridium perfringens*, in temperature-abused foods accounts for one of the most common causes of foodborne illness in the United States (Mead and others 1999). Although it can be assumed that at least 50% of all raw meat products contain 10^3 to 10^4 viable *C. perfringens* colony-forming units (CFU)/g, typical disease symptoms of diarrhea and abdominal pain are only associated with ingested food contaminated with greater than 10^6 viable cells/g (Hobbs 1979; Labbe 1981; McClane 1997). Only from such a dose will enough vegetative cells survive the gastrointestinal environment to sporulate in the small intestine and induce illness (Labbe and Juneja 2002). Furthermore, sporulation and enterotoxin (*C. perfringens* enterotoxin [CPE]) production in *C. perfringens* have been shown to increase concomitant with increases in cell density (Garcia-Alvarado and others 1992).

Quorum sensing, or cell-to-cell signaling as a result of bacterial population density, has been shown to regulate various pathogen virulence processes such as antibiotic production, biofilm formation, sporulation, and toxin production (Brelles-Marino and Bedmar 2001; Miller and Bassler 2001; Schauder and Bassler 2001). This communication at the cellular level, based on accumulation of chemical signaling molecules called autoinducers, was 1st described in the regulation of bioluminescence in the marine symbiont, *Vibrio fischeri* (Hastings and Nealson 1977; Nealson and Hastings 1979). Since then, Gram-negative bacteria have been found to respond to a range of acylated homoserine lactone autoinducers, designated autoinducer-1 (AI-1) (McClean and others 1997; Surette and Bassler 1998; Schauder and others 2001), whereas Gram-positive bacteria rely on a series of processed oligopeptides for intraspecies cellular communication (Kleerebezam and Quadri 2001; Miller and Bassler 2001; Schauder and Bassler 2001). A 3rd type of autoinducer, designated autoinducer-2 (AI-2), is presumed to be used by bacteria as a universal communication signal among and between different species of bacteria (Schauder and Bassler 2001; Cloak and others 2002; Malott and Lo 2002). The *luxS* gene, found in numerous bacteria, is necessary for the synthesis of AI-2, and there is sufficient evidence to believe that AI-2 is likely a furanone derivative or metabolite (Chen and others 2002; Elvers and Park 2002; Winzer and others 2002).

In *C. perfringens*, a *luxS* mutant was constructed, which showed measured reductions in extracellular θ-, κ-, and α-toxin levels, although CPE levels were never tested (Ohtani and others 2002). Foodborne illness is exclusively dependent upon CPE levels in the intestines. Preformed CPE, however, in foods is not generally implicated in foodborne illness and heating for 5 min at 60 °C inactivates this enterotoxin (McClane 1997). Cooking of foods not only destroys the enterotoxin but also decreases the numbers of vegetative cells capable of subsequently forming spores. Still *C. perfringens* in foods remains a significant cause of foodborne illness. Because *C. perfringens* regulates cellular processes through production of AI-2, inhibition of growth and sporulation of *C. perfringens* on raw food products may be possible through the use of quorum-sensing analogues. Natural and synthetic analogues of autoinducers have been identified and used to inhibit biofilm formation and swarming in bacterial populations through inhibition of quorum sensing (Manefield and others 2002; Ren and others 2002; Reverchon and others 2002). Because naturally occurring furanones, such as 5-(1,2-dihydroxyethyl)-3,4-dihydroxy-2(5H)-furanone (ascorbic acid) or 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF), are found in certain fruits and vegetables (Slaughter 1999), these molecules could be exploited to control quorum sensing in bacteria in different types of food. This study represents the 1st use of natural furanones to examine the effect on growth, sporulation, and CPE production in *C. perfringens* and AI-2 production in food.

Materials and Methods

Bacterial strains and chemicals

The strains used in this study are listed in Table 1. Only enterotox-

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Table 1—Bacterial strains used in this study

Species/strain	Description	Reference (source)
Vibrio harveyi BB170[a]	Bioluminescence assay reporter strain (sensor1$^-$, 2$^+$)	Surette and Bassler 1998
Clostridium perfringens B40[b]	Human origin, Europe, 1980s Plasmid *cpe* gene	Sarker and others 2000
C. perfringens E13[b]	United States, 1960s Chromosomal *cpe* gene	Sarker and others 2000
C. perfringens F5603[b]	Human origin, Europe, 1980s Plasmid *cpe* gene	Sarker and others 2000
C. perfringens FD1041[b]	United States, 1980s Chromosomal *cpe* gene	Sarker and others 2000
C. perfringens H6	Hobbs serotype 6 NCTC 8679	CDC[c]

[a]Strain kindly provided by Dr. Bonnie L. Bassler of Princeton Univ., Princeton, N.J.
[b]Strains kindly provided by Dr. Bruce A. McClane of the Univ. of Pittsburgh, Pittsburgh, Pa.
[c]CDC = Centers for Disease Control and Prevention, Atlanta, Ga.

in-producing strains of *C. perfringens* were used. Isolates linked to foodborne illness contained the chromosomal *cpe* gene, whereas isolates not associated with foodborne disease contained a plasmid *cpe* gene. A luminescent reporter strain of *Vibrio harveyi,* capable of sensing AI-2 but not AI-1, was used to monitor production of AI-2 in *C. perfringens* from a synthase encoded by the *luxS* gene. Growth media, including Luria-Bertani (LB), fluid thioglycollate (FTG), and brain heart infusion (BHI) broth and peptone, were purchased from Difco Laboratories (Detroit, Mich., U.S.A.). All biochemicals used for molecular analyses were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A.). All commercially obtained furanones were from Fluka Laboratory Chemicals (Milwaukee, Wis., U.S.A.). Protein molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, Calif., U.S.A.). Rabbit antiserum raised against CPE was provided as a generous gift from Dr. Bruce A. McClane (Univ. of Pittsburgh, Pittsburgh, Pa., U.S.A.).

AI-2 bioassay

The measurement of autoinducer by reporter cells was assayed in autoinducer bioassay (AB) medium (Greenberg and others 1979) as relative light units (RLU) produced from cell-free supernatants as described by Surette and Bassler (1998) with the following modifications: Wallac B & W 96-well isoplates (Perkin Elmer Life Sciences, Boston, Mass., U.S.A.) were used to decrease crosstalk between sample wells. The Wallac Victor^2 (Perkin Elmer) was used with orbital mixing of samples every 20 min before readings were recorded during the 6-h duration of assay measurements at 30 °C. The RLU were expressed as total luminescence per 10^6 *Vibrio harveyi* strain BB170 cells/well. Background corrections were calculated by subtracting the control RLU values (reporter strain + 10% test medium) from the experimental RLU values (reporter strain + 10% test medium from samples inoculated with *C. perfringens*) obtained for each sample variable and run. Measurements of 3 replicate samples were taken and averaged to obtain mean values ± standard deviations. All experiments were performed 2 to 3 times.

Growth of a 5-strain cocktail on ground beef

C. perfringens culture stocks frozen in 20% glycerol and stored at –80 °C were streaked onto BHI agar plates and then incubated at 32 °C anaerobically (85% N_2, 10% CO_2, 5% H_2) for 18 h. The 5 strains of *C. perfringens* (Table 1) were grown individually in tubes containing 10 mL of FTG medium at 37 °C for 6 to 8 h. Following growth at 37 °C, the cultures were combined in equal volumes and mixed to form an inoculum (10^9 CFU/mL) cocktail in a sterile 50-mL centrifuge tube. Ground beef (93% lean; purchased from a local supermarket) was placed in a foil-covered beaker (200 g) to which 200 mL dH_2O were added and autoclaved (15 psi at 121 °C for 15 min). Upon sterilization, the cooked meat was separated from liquid runoff and dispensed aseptically in 10-g portions into sterile filter-lined Stomacher bags (Nasco, Fort Atkinson, Wis., U.S.A.). One milliliter of culture cocktail was added, followed by mixing by hand, and the bags were individually vacuum-sealed to 20 mbar using a vacuum packager (Multi-Vac Inc., Model A300, Kansas City, Mo., U.S.A.). All of the samples were then incubated at 37 °C for 0, 6, 24, and 48 h. Vacuum bags containing the ground beef samples were opened at prescribed incubation times, 10 mL of 0.1% (w/v) peptone water (PW) was added, and the bags were hand-massaged for 30 s followed by 2 min of pummeling using a Stomacher lab-blender (Model 400, Spiral Systems, Inc., Cincinnati, Ohio, U.S.A.). Suspended bacteria were spread-plated following dilution in 0.1% PW onto BHI agar plates, then incubated at 37 °C under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2) for 16 to 20 h. Duplicate 1-mL samples were centrifuged, filtered as described previously, and stored at –20 °C until assayed for AI-2 or pH. Medium supernatant pH was measured using a Corning pH meter with pH combination electrode nr 430 (Corning Inc. Science Products Div., Corning, N.Y., U.S.A.). Supernatants were tested for AI-2 as described previously using the AI-2 bioassay. All experiments were performed at least twice with 3 replicates per experiment, and means were plotted ± standard deviations.

Furanone interference with AI-2 production in supernatants from ground beef

To measure the effects of compounds structurally similar to AI-2 on light production using the *V. harveyi*-coupled assay, 10 μL of each commercially obtained furanone (ascorbic acid, sodium ascorbate, 4-hydroxy-2[*5H*]-furanone [HF], 4,5-dihydro-2-methyl-3[*2H*]-furanone [DHMF], 4-hydroxy-2,5-dimethyl-3[*2H*]-furanone [DMHF], and L-homoserine lactone hydrobromide [HSL-HBr]) at concentrations of 0, 10, 30, and 100 m*M* were combined 1:1 with cell-free supernatants obtained from ground beef inoculated and incubated with *C. perfringens* as described previously. The subsequent effect of each furanone on RLU per 10^6 *V. harveyi* BB170 cells was measured using the AI-2 bioassay and evaluated. pH measurements were determined to evaluate ascorbic acid and sodium ascorbate effects on AI-2 production.

Ground beef was autoclaved and prepared as previously described in this study. The liquid runoff was discarded. Before the culture cocktail was added, ascorbic acid (vitamin C) in a 1-mL aqueous volume was added directly to final concentrations of 0 (control), 10, 30, and 300 m*M* per 10-g portions of the cooked beef in vacuum filter bags (Nasco) that were then hand-mixed for 1 min. One milliliter of the 5-strain *C. perfringens* culture cocktail was then added as described previously and mixed with the beef, and the bags were vacuum-sealed to 20 mbar using a vacuum packager. The samples were then incubated at 37 °C for 0, 1, 2, 4, and 7 d. As before, vacuum filter bags containing the ground beef samples were opened at prescribed incubation times, 10 mL PW were added, and the bags were hand-massaged for 30 s followed by 2 min of pummeling using a

Stomacher laboratory blender. Viable cells were spread-plated following dilution onto BHI agar plates, then incubated at 37 °C under anaerobic conditions for 16 to 20 h. Duplicate samples were centrifuged (16000 × *g* for 5 min) followed by filtration using 0.2-μm syringe filters and storage at –20 °C until assayed for AI-2. Supernatants were tested for AI-2 as described previously for the AI-2 bioassay. All experiments were performed at least twice with 3 replicates per experiment, and means were plotted ± standard deviations.

Effect of ascorbic acid on sporulation of *C. perfringens*

Individual cultures of the 5 *C. perfringens* strains (Table 1) grown for 18 h in FTG were transferred to fresh FTG tubes and grown for 4 h at 37 °C. The 5 actively growing cultures were mixed to form a log phase culture cocktail (approximately 7.6 $\log_{10}$ CFU/mL). Next, 200 μL of the culture were added to tubes containing 10 mL of Duncan and Strong (DS) sporulation medium (Duncan and Strong 1968) prepared with 0.4% (w/v) raffinose (Sigma-Aldrich Chemical Co.) in place of starch (Labbe and Rey 1979) and 0.5 m*M* caffeine (Juneja and others 1993), and filtered ascorbic acid (1 *M*) or sodium ascorbate (1 *M*) were added to final concentrations of 10, 30, and 100 m*M*. The samples were then incubated for 0, 4, 24, 48, and 72 h at 37 °C. Following dilution, viable cells were spread-plated onto BHI agar plates and then incubated at 37 °C under anaerobic conditions for 16 to 20 h. Duplicate samples were centrifuged (16000 × *g* for 5 min) followed by filtration using 0.2-μm syringe filters and storage at –20 °C until assayed for AI-2. Samples were also collected for pH measurements. Supernatants were tested for AI-2 as described previously, using the AI-2 bioassay. One milliliter of the DS samples in microcentrifuge tubes were immersed in a 75 °C water bath for 20 min to kill all vegetative cells. Spores were plated onto BHI agar and grown under anaerobic conditions for 16 h at 37 °C. The presence of spores in cultures before plating was verified using the Schaeffer-Fulton endospore stain (Schaeffer and Fulton 1933) followed by microscopic examination. All experiments were performed at least twice with 3 replicates per experiment and means were plotted ± standard deviations.

Effect of ascorbic acid on CPE production

One milliliter samples from the DS medium inoculated with the *C. perfringens* 5-strain cocktail and with varying ascorbic acid or sodium ascorbate concentrations as previously described were centrifuged (16000 × *g*). Pellets were resuspended in 0.5 mL lysis buffer (Rheinberger and others 1988). Samples containing spores and cells were disrupted on ice using a Vibracell Ultrasonic Processor (Model VC130, Sonics & Materials, Inc., Newtown, Conn., U.S.A.) at an amplitude setting of 60 and a 1-s pulse for 2 min. The total protein in the samples was determined using a modification of the Lowry procedure (Lowry and others 1951; Markwell and others 1978). Proteins were subjected to denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (4% [w/v] acrylamide stacking gel, 15% [w/v] acrylamide resolving gel; 30:0.8 acrylamide: bis acrylamide) using a modified Tris-glycine buffer system (Lugtenberg and others 1975). Denatured proteins were transferred to Immobilon-P polyvinylidene difluoride membranes (Fisher Scientific, Pittsburgh, Pa., U.S.A.) using a Panther semidry electroblotting apparatus model HEP-1 (Owl Separation Systems, Portsmouth, N.H., U.S.A.). Blots were prewashed with phosphate-buffered saline (PBS) 0.05% (v/v) Tween-20 (PBS-Tween) (Sambrook and others 1989) and were incubated overnight in dehydrated nonfat milk (5%) (w/v) in PBS-Tween as a blocking agent. Polyclonal rabbit antiserum raised against the *C. perfringens* enterotoxin (CPE) diluted in PBS-Tween (1:3000) was added to the blots that were then incubated with agitation for 1 h at ambient temperature (Novak and Tabita 1999). A colorimetric detection assay was used with the secondary antibody consisting of goat anti-rabbit IgG conjugated to alkaline phosphatase (Gibco BRL Life Technologies, Grand Island, N.Y., U.S.A.) that was diluted 1:3000 in PBS-Tween and incubated for 2 h earlier for chromogenic substrate development (Sambrook and others 1989).

Results and Discussion

Quorum sensing is defined as communication within or between species of bacteria resulting in alterations in gene expression through the use of small chemical signaling molecules called autoinducers produced as a function of cell density and environmental conditions (Miller and Bassler 2001). Before knowledge of quorum sensing, growth, and sporulation in *C. perfringens* were reportedly influenced by exogenously added enterotoxin (Dillon and Labbe 1989). Addition of culture fluids has also been shown to promote sporulation in *C. perfringens* attributable to small-molecular-weight factors resistant to alkali or acids, protein denaturing temperatures, and proteases (Shih and Labbe 1996; Tseng and Labbe 2000). The gene locus, *luxS*, encoding the AI-2 synthase necessary for AI-2 production, was recently sequenced from *C. perfringens* and shown to be involved in toxin production (Schauder and others 2001; Ohtani and others 2002; Shimizu and others 2002). Although posttranslationally modified peptides have been described to regulate cell-to-cell communication in Gram-positive bacteria (Bassler 1999; Schauder and Bassler 2001), signaling through AI-2 was chosen for study after proven applicability of a coupled bioluminescence assay for detection of AI-2 (Surette and Bassler 1998; Schauder and others 2001; Cloak and others 2002).

Various *C. perfringens* enterotoxin-producing strains (Table 1) were individually tested for comparisons of culture growth and increases in *V. harveyi* reporter strain bioluminescence from their respective cell-free supernatants. Growth and AI-2 production measurements in LB medium were similar for each of the strains tested

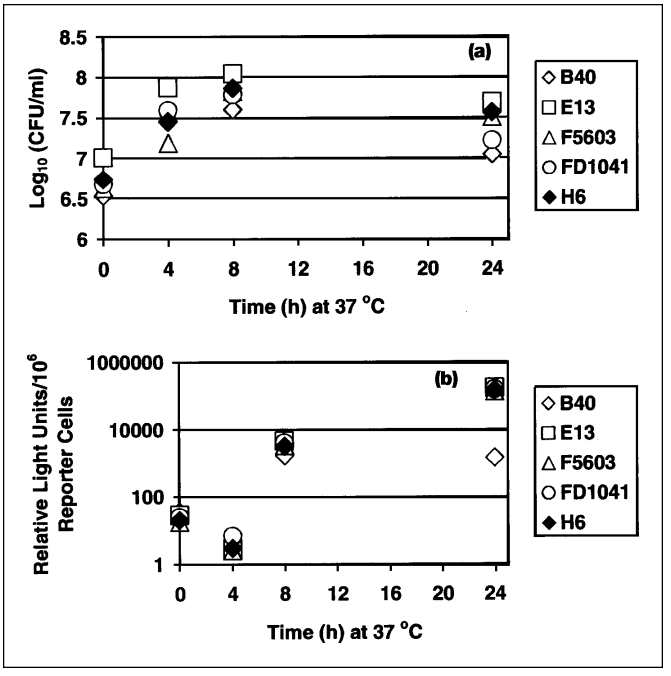

Figure 1—(a) Growth of *Clostridium perfringens* strains in Luria-Bertani (LB) medium. (b) Autoinducer-2 (AI-2) production measured in relative light units (RLU) over the same time period.

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(Figure 1). The greatest differences in sample RLUs compared with control values were found following 48 h growth at 37 °C on ground beef. Consequently, all furanone analogues were initially tested in cell-free supernatants from ground beef cultures held at 37 °C for 48 h and compared following AI-2 bioassay conditions as described in the Materials and Methods section.

A number of commercially available furanone compounds, structurally similar to AI-2 precursors, were examined for their ability to stimulate or inhibit reporter activity in the *V. harveyi* BB170 bioassay (Figure 2). The AI-2 activities of supernatants from the *C. perfringens* cocktail grown on ground beef for 48 h at 37 °C were measured using the reporter bioassay following the addition of each furanone at 0-, 10-, 30-, and 100-m*M* concentrations. Increasing ascorbic acid concentrations (10 to 100 m*M*) resulted in RLU decreases using the bioassay (Figure 2). Levels of AI-2 in the presence of 30 or 100 m*M* ascorbic acid were inhibited to the same extent regardless of the presence of *C. perfringens*. The different furanones may compete with AI-2 for select *V. harveyi* receptor sites resulting in the exhibited RLU decreases. Thus, increasing concentrations of ascorbic acid, sodium ascorbate, and HF resulted in similar decreases in RLUs by competition with AI-2 for available receptor sites in the reporter strain (Figure 2). The background (no ascorbic acid) was not subtracted for the results in Figure 2 because the inhibition in AI-2 production caused by furanone analogues would have resulted in negative values. The addition of DHMF and HSL-HBr resulted in higher RLU values with increasing furanone concentrations compared with controls (data not shown). Also, DHMF resulted in increases of lower magnitude than those of DHMF and HSL-HBr with increasing dose with respect to AI-2 activity (unpublished data). On the basis of these results, ascorbic acid was selected to further evaluate its ability to potentially inhibit quorum-sensing (AI-2 activity) effects on *C. perfringens* growth, sporulation, and enterotoxin production. Sodium ascorbate was included to determine the contributions of medium acidification by ascorbic acid.

Ascorbic acid was added directly to ground beef at 10-, 30-, and 300-m*M* concentrations before inoculation with a 5-strain mixture of *C. perfringens* (10^4 to 10^5 CFU/mL). The levels of ascorbic acid used in this study were consistent with those used in another study

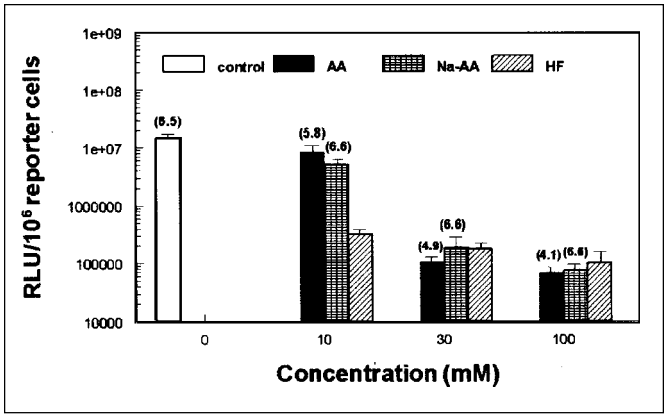

Figure 2—The concentration effects of commercially available furanones found to inhibit autoinducer-2 (AI-2) measurements using the *Vibrio harveyi* bioluminescence assay and culture supernatants from a *Clostridium perfringens* 5-strain cocktail grown on ground beef for 48 h. Error bars depict the standard deviations for means of 3 replicates. Supernatant pH values are enclosed in parentheses for ascorbic acid or sodium ascorbate additions. Control = no furanone additions; AA = ascorbic acid; Na-AA = sodium ascorbate; HF = 4-hydroxy-2(*5H*)-furanone.

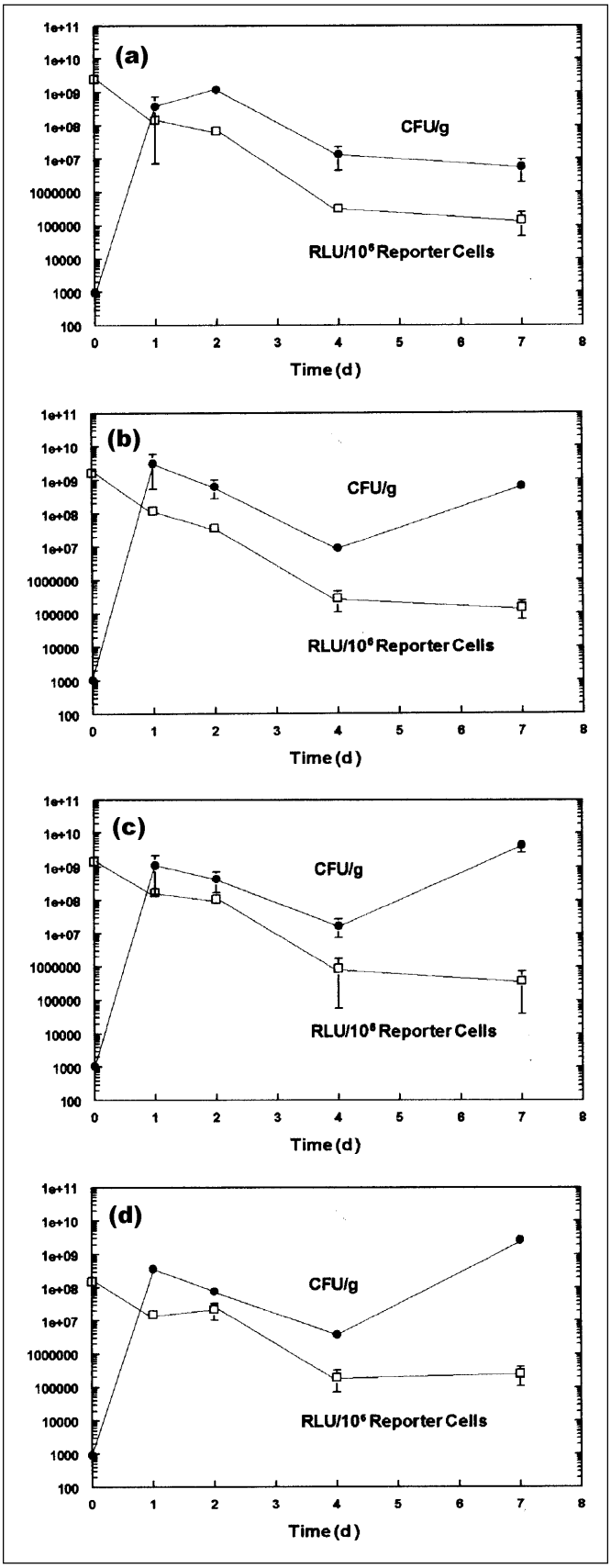

Figure 3—Growth of the 5-strain cocktail of *Clostridium perfringens* on ground beef and corresponding supernatant autoinducer-2 (AI-2) measurements over 7 d. The following ascorbic acid additions to ground beef were made: (a) 0 m*M*, (b) 10 m*M*, (c) 30 m*M*, and (d) 300 m*M*.

examining the antimicrobial properties of ascorbic acid combined with gamma irradiation in ground beef (Ouattara and others 2002). Results of AI-2 activity were different than those obtained when ascorbic acid was added directly to supernatants from *C. perfringens*-inoculated ground beef as described in the preceding section. The growth of *C. perfringens* was relatively unchanged by additions of ascorbic acid to ground beef (10, 30, and 300 m*M* compared with no ascorbic acid), except after 4 d, when viability was enhanced in the presence of ascorbic acid (Figure 3). There was a steady decrease in AI-2 activity over the course of the 7-d incubation at 37 °C in ground beef with and without the addition of ascorbic acid (Figure 3). The observed decreases in AI-2 activity over time were likely associated with the accumulation of metabolic waste products. A pH decrease in growth medium pH below 5.0 would be expected to inhibit cellular growth (DeJong 1989) and resultant AI-2 production by *C. perfringens*. Some of these metabolic products were possibly degraded through a secondary metabolite mechanism represented by the biphasic growth curves in the presence of ascorbic acid (Figure 3). For the ground beef samples inoculated with *C. perfringens*, increasing concentrations of ascorbic acid (10 to 300 m*M*) stimulated a return to high cell numbers (about 9 to 10 $\log_{10}$ CFU/g) after 7 d of growth, which was represented by a biphasic growth curve and possible metabolism of the ascorbic acid (Figure 3).

Only ascorbic acid at the highest concentration tested (300 m*M*) affected the measured AI-2 starting levels at day 0 in ground beef, causing at least a 1-$\log_{10}$ decrease (Figure 3). This inhibitory effect on AI-2 activity by ascorbic acid was observed using inoculated ground beef (Figure 3), supernatants from the inoculated beef (Figure 2), or supernatants from DS medium inoculated with *C. perfringens*. Some of the metabolic products were possibly degraded through a secondary metabolite mechanism represented by the biphasic growth curves in the presence of ascorbic acid (Figure 3). However, AI-2 activity did not return to higher levels with the increase in cell numbers.

The addition of 10 to 30 m*M* ascorbic acid or sodium ascorbate to sporulation medium resulted in decreased growth for *C. perfringens* following 48 h incubation at 37 °C as compared with a control without any added furanone analogues (data not shown). The AI-2 activity also decreased over the same time period in the presence of ascorbic acid or sodium ascorbate. Differences because of pH were pronounced with a 100-m*M* concentration of ascorbic acid (pH 4.0), resulting in complete inactivation of *C. perfringens*, whereas 100 m*M* sodium ascorbate (pH 7.4) resulted in growth comparable to samples containing 30 m*M* sodium ascorbate (data not shown).

Low values for AI-2 activity representative of reporter cell background values were reached in the presence of both ascorbic acid and sodium ascorbate at 100 m*M*. Use of sodium ascorbate in place of ascorbic acid resulted in a similar inhibition of AI-2 activity as ascorbic acid without influencing bioassay medium pH (Table 2). As metabolic products likely accumulated in the DS medium from cellular growth over 24 h, there was a steady decrease in viable cell counts with increasing ascorbic acid concentrations that also might have impacted growth. The medium pH, however, had little effect on the AI-2 bioassay pH. except at a 100 m*M* ascorbic acid concentration (Table 2). The AI-2 activity in DS medium decreased before a recorded decrease in cell numbers for *C. perfringens*. It is important to stress the need for careful evaluation of each growth medium or food source to be tested along with appropriate background corrections before any assumptions can be made regarding autoinducer activities using this coupled bioassay.

Spore production by *C. perfringens* was determined in DS medium because this medium afforded greater reproducibility in spore production than ground beef. The total spore production for *C. perfringens* decreased significantly ($P < 0.05$) in the presence of added ascorbic acid (10 or 30 m*M*) in DS medium. The medium pH was found to drop from 7.4 to 6.8 and 5.5 with 10 and 30 m*M* ascorbic acid, respectively (Table 2). Likewise, a recent study reported the effects of various sporulation-promoting substances and concluded that acid conditions did not increase sporulation of *C. perfringens* (DeJong and others 2002). Ascorbic acid concentrations of 100 m*M* prevented sporulation, likely by inhibiting *C. perfringens* growth because of a culture medium pH reduction to 4 (Table 2). Sodium ascorbate did not influence spore levels nor did it result in decreased medium pH levels (Table 2). Spore formation in *C. per-*

Table 2—Total spore production after 48 h at 37 °C in Duncan-Strong medium

Ascorbic acid (m*M*)	Spores ($\log_{10}$/mL)[a]	Sporulation (%) total	Initial culture medium pH[b]	AI-2 assay medium pH[b]
0	6.83 ± 0.36	79.92 ± 7.76	7.4	6.9
10	1.57 ± 0.67	27.26 ± 9.87	6.8	6.6
30	3.14 ± 0.79	59.14 ± 12.89	5.5	6.3
100	BDL[c]	BDL[c]	4.0	4.8
Na-ascorbate (m*M*)	**Spores ($\log_{10}$/mL)[a]**	**Sporulation (%) total**	**Initial culture medium pH[b]**	**AI-2 assay medium pH[b]**
0	6.83 ± 0.36	79.92 ± 7.76	7.5	6.9
10	4.41 ± 1.21	73.97 ± 21.53	7.5	6.9
30	4.43 ± 1.17	77.17 ± 18.96	7.4	6.8
100	4.38 ± 1.17	75.60 ± 17.44	7.4	6.9

[a]1-mL samples of culture (in triplicate) were taken and immersed in a 75 °C water bath for 20 min. Spore totals were counted as the mean viable $\log_{10}$ colony-forming units (CFU)/mL ± standard deviations after 16 h of growth on brain heart infusion (BHI) medium under anaerobic conditions (85% N_2, 10% CO_2, 5% H_2).
[b]Medium pH was measured using a Corning pH meter with pH combination electrode nr 430 (Corning Inc. Science Products Div., Corning, N.Y., U.S.A.).
[c]Below detectable limits (<10 CFU/mL) under the conditions used for the experiment. The absence of spores was verified using the Schaeffer-Fulton endospore stain (Schaeffer and Fulton 1933) followed by microscopic examination.

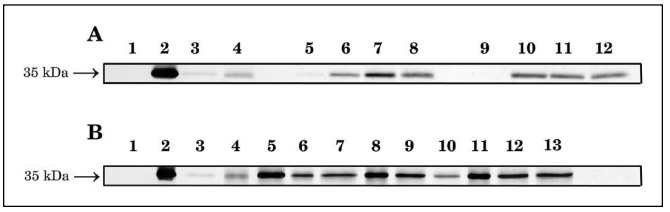

Figure 4—Western immunoblots of sodium dodecyl sulfate-polyacrylamide electrophoresis gels containing crude cell lysates from *Clostridium perfringens* cells grown in Duncan and Strong (DS) medium in the presence of varying (a) ascorbic acid or (b) sodium ascorbate concentrations over time. Blots were reacted against polyclonal antiserum raised against *C. perfringens* enterotoxin (CPE). Ten micrograms of total protein were loaded in each well. Arrows indicate location of the 35-kDa antigen. Ascorbic acid concentrations and time of cell harvest in (a): Lanes (1) 0 m*M* after 4 h; (2) 0 m*M* after 24 h; (3) 0 m*M* after 48 h; (4) 0 m*M* after 72 h; (5) 10 m*M* after 4 h; (6) 10 m*M* after 24 h; (7) 10 m*M* after 48 h; (8) 10 m*M* after 72 h; (9) 30 m*M* after 4 h; (10) 30 m*M* after 24 h; (11) 30 m*M* after 48 h; and (12) 30 m*M* after 72 h. Sodium ascorbic acid concentration and time of cell harvest in (b): Lanes (1-4) same as lanes 1-4 in (a) above; (5) 10 m*M* after 24 h; (6) 10 m*M* after 48 h; (7) 10 m*M* after 72 h; (8) 30 m*M* after 24 h; (9) 30 m*M* after 48 h; (10) 30 m*M* after 72 h; (11) 100 m*M* after 24 h; (12) 100 m*M* after 48 h; and (13) 100 m*M* after 72 h.

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fringens has been found to closely follow growth (Garcia-Alvarado and others 1992), and growth of *C. perfringens* only rarely occurs at a pH <5.0 (DeJong 1989). Although discounted by the results obtained with sodium ascorbate in the present study, the inhibition of sporulation could still be linked to quorum sensing and to interference of AI-2 receptor sites. Only levels of ascorbic acid equivalent to 100 m*M* caused a significant drop in the pH of the AI-2 bioassay medium to pH 4.8. Therefore, inhibition of the *V. harveyi* reporter strain by pH is likely not a consideration.

The effects of ascorbic acid on CPE production in *C. perfringens* in DS medium were also examined. In the absence of ascorbic acid, an antioxidant, CPE levels were highest following 24-h incubation in the sporulation medium (Figure 4a). Afterward, CPE declined, possibly because of degradation or metabolic turnover events. However, in the presence of 10 to 30 m*M* ascorbic acid, CPE levels, although lower than controls without ascorbic acid additions, were maintained for up to 72 h incubation at 37 °C. Sodium ascorbate also delayed CPE turnover compared with controls. Acidic conditions can conceivably increase toxin conformational stability and inhibit degradative proteases as well (Figure 4b). These findings were unexpected, and it is possible that the presence of ascorbic acid, although interacting with AI-2 activity in an undetermined manner, may help to stabilize toxin production. Ascorbic acid may prevent the degradation of CPE by binding to catabolic proteases that are produced by *C. perfringens*. Further studies in this area are under way to verify this finding.

Conclusions

AI-2 activity was measured for *C. perfringens* using the *V. harveyi*-coupled bioluminescent assay. AI-2 precursor analogues, such as ascorbic acid, sodium ascorbate, and HF, exhibited negative effects on AI-2 activity in *C. perfringens* as well as in the *V. harveyi* reporter strain. The current study is the 1st to report the effects of a natural quorum-sensing analogue, when added to ground beef, on the activity of an interspecies signaling compound (AI-2), on sporulation, and on CPE production in *C. perfringens*. The antioxidant properties of ascorbic acid possibly led to the preservation and stabilization of the enterotoxin produced by *C. perfringens*. Ascorbic acid was shown in this study to interfere with the AI-2 activity of a foodborne pathogen. Because of the chemical complexity of food environments, interference from multiple metabolites can affect cell-to-cell signaling in microorganisms. The results of this study support the need for further evaluation of potential quorum-sensing inhibitor molecules for effects on pathogen growth, toxin production, and biofilm formation in foods.

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